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Research Article

# Two new species of *Colletotrichum* (Glomerellaceae, Glomerellales) causing walnut anthracnose in Beijing

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## Abstract

*Colletotrichum* species are plant pathogens, saprobes and endophytes on various plant hosts. It is regarded as one of the 10 most important genera of plant pathogens in the world. Walnut anthracnose is one of the most severe diseases affecting walnut productivity and quality in China. In this study, 162 isolates were obtained from 30 fruits and 65 leaf samples of walnut collected in Beijing, China. Based on morphological characteristics and DNA sequence analyses of the concatenated loci, namely internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin (*ACT*), chitin synthase 1 (*CHS-1*) and beta-tubulin (*TUB2*), these isolates were identified as two novel species of *Colletotrichum*, i.e. *C. juglandicola* and *C. peakense*. Koch's postulates indicated that both *C. juglandicola* and *C. peakense* could cause anthracnose in walnut.

**Key words:** Anthracnose, multi-gene phylogeny, pathogenicity, walnut

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## Introduction

Walnut (*Juglans regia* L.), a deciduous tree, is an essential woody nut and oil crop cultivated worldwide (Da Lio et al. 2018). Walnut fruit is rich in linolenic acid and lacking cholesterol and ranks first amongst the world's "four major dried fruits" (Pei and Lu 2011). Walnut cultivation in China has a history of more than two thousand years and is China's "woody grain and oil" strategic tree species (Guo 2016; Liu et al. 2021). The walnut productivity in China contributed 47% of the global production in 2017 and ranked first worldwide since 2017 (Liu et al. 2021).

*Colletotrichum* Corda (Glomerellaceae, Glomerellales, Sordariomycetes) was introduced, based on the morphological feature of the conidiomata with setae and *Colletotrichum lineola* Corda was assigned as the generic type (Corda 1831). The sexual morph of *Colletotrichum* was previously known as the genera *Gnomoniopsis* and *Glomerella* (Stoneman 1898; von Schrenk and Spaulding 1903; Marin-Felix et al. 2017). With the implementation of "one fungus one name" nomenclature, *Colletotrichum* has been chosen to represent this genus, based on priority (Réblová et al. 2016). *Colletotrichum* was characterised by acervular conidiomata, often with setae, producing cylindrical or crescent-shaped conidia and by the formation of appressoria (Sutton 1992;

Marin-Felix et al. 2017). More than 1,000 epithets have been accommodated within *Colletotrichum* (http://www.indexfungorum.org, accessed 20 March 2023), while about 300 species have DNA sequence data to support their taxonomic status within *Colletotrichum*. Sixteen species complexes have been recognised within *Colletotrichum*, with *C. gloeosporioides* species complex as the largest one, which occupies more than 18% of all the recognised taxa of *Colletotrichum* (Marin-Felix et al. 2017; Damm et al. 2019; Jayawardena et al. 2020; Bhunjun et al. 2021; Mu et al. 2021; Talhinhas and Baroncelli 2021; Alizadeh et al. 2022; Liu et al. 2022; Tsushima and Shirasu 2022; Zheng et al. 2022).

*Colletotrichum* spp. comprised important plant pathogens, while others are endophytes or saprobes (Cannon et al. 2012; Hyde et al. 2014; Jayawardena et al. 2016). Some *Colletotrichum* species have been reported causing anthracnose disease on various host plants (Hyde et al. 2014; Nilsson et al. 2014). For instance, the causal agents of ginseng anthracnose were *C. lineola* and *C. panacicola* in China (Liu et al. 2020). Anthracnose of *Pyrus* spp. was caused by 12 *Colletotrichum* species in China, viz. *C. aenigma*, *C. citricola*, *C. conoides*, *C. fioriniae*, *C. fructicola*, *C. gloeosporioides*, *C. karstii*, *C. plurivorum*, *C. siamense*, *C. wuxiense*, *C. jinshuiense* and *C. pyrifoliae* (Fu et al. 2019). Quite a few species of *Collectotrichum* have been reported to be causing walnut anthracnose disease, which has resulted in a considerable reduction in walnut production worldwide (Zhu et al. 2014; Wang et al. 2016; Da Lio et al. 2018). For instance, the causal agent of walnut anthracnose identified as *Colletotrichum* spp. led to 50–70% losses, with some walnut orchards experiencing 100% losses in nut production in France (Giraud and Verhaeghe 2015). *Colletotrichum nymphaeae* caused anthracnose in walnut in Brazil, destroyed approximately 50% of the fruits and the incidence was higher in rainy and hot summers (Savian et al. 2019).

In China, 12 *Colletotrichum* species have been reported causing walnut anthracnose. Sever walnut anthracnose occurred in the orchards of Shandong Province, with the causal agents *C. gloeosporioides* sensu lato, *C. siamense*, *C. fructicola* and *C. viniferum* (Zhu et al. 2014; Wang et al. 2017, 2018; He et al. 2019). The walnut leaf anthracnose caused by *C. fioriniae* led to severe loss in nut production in Hechi, Guangxi Province (Zhu et al. 2015). In addition, *Colletotrichum aenigma* caused severe fruit anthracnose in Hebei Province (Wang et al. 2021). *Colletotrichum nymphaeae* caused walnut branches anthracnose in Gansu Province (Ma et al. 2022). *Colletotrichum gloeosporioides*, *C. kahawae*, *C. nymphaeae*, *C. godetiae*, *C. fioriniae* and *C. juglandis* caused leaf spots of walnut in Hubei Province (Wei et al. 2022). *Colletotrichum godetiae* caused severe anthracnose of walnut in Shaanxi Province and Yunnan Province with diseased fruits over 60% in the orchard (Wang et al. 2023). Li et al. (2023) collected 900 walnut leaves and 300 fruits samples from seven districts of Beijing and 377 isolates of *Colletotrichum* spp. were identified into six species, namely *C. aenigma*, *C. fructicola*, *C. gloeosporioides*, *C. siamense*, *C. liaoningense* and *C. sojae*. All of these six species caused anthracnose of walnut and *C. gloeosporioides* showed the highest virulence.

In the course of an ongoing survey of pathogenic fungi of walnuts in China initiated in 2021, the symptoms on the fruits included round brown spots in the early stage that later turned black. As environmental humidity increased, the spots were covered with orange-red conidiomata. Some spots were merged into large necrotic areas, causing the whole fruit to blacken and rot, resulting in fruit drop. The

symptoms on the leaves included nearly round or irregular black or brown spots and gradually withering. A total of 162 isolates were obtained from 30 fruits and 65 leaf samples of walnut collected in the suburb area of Beijing. Their taxonomic status was evaluated, based on morphological characteristics and DNA sequence comparisons and pathogenicity were evaluated by proving Koch's postulates.

## Materials and methods

### Sample collection and fungal isolation

Thirty fruits and sixty-five leaf samples exhibiting anthracnose were collected from the suburb area of Beijing, China, in August, 2021. Specimens were transferred to the laboratory and kept in a freezer. Fragments (0.5 × 0.5 cm) were cut aseptically from the margin of the disease lesion and surface-sterilised with 75% ethanol for 30 s, rinsed three times with sterile distilled water, dried on sterilised filter paper and incubated on malt extract agar (MEA; 2%) for isolation of fungal strains (Zhao et al. 2019a). Petri dishes were incubated in the dark at 25 °C until the fungal colonies were observed. Hyphal tips resembling *Colletotrichum* colonies were transferred to Petri dishes with MEA. Isolates grown on MEA in the dark were kept at 25 °C to determine colony characteristics.

### Morphological characterisation

To assess the colony characteristics, mycelial plugs (8 mm in diameter) were transferred from the growing edges of 7-day-old colonies on to PDA and MEA and incubated at 25 °C under dark conditions (Liu et al. 2017a; Zhao et al. 2019a). Colony diameters were measured after 7 days' incubation (Liu et al. 2017b) and were used to calculate hyphal growth rate (Mo et al. 2018). Morphology and colony characteristics were determined following Damm et al. (2009, 2014) and Choi et al. (2011). Appressoria were induced on slide cultures, according to Weir et al. (2012). The shape, colour and size of conidia, conidiophores, setae, conidiogenous cells and appressoria were measured by at least 20 measurements using a microscope (Nikon Eclipse E600) (Zhao et al. 2019b). Fungal isolates and specimens were deposited at Beijing Forestry University, with duplicates at the China General Microbiological Culture Collection Center (**CGMCC**) and the Mycological Herbarium of the Institute of Microbiology, Chinese Academy of Sciences (**HMAS**).

### DNA extraction, PCR amplification and sequencing

DNA was extracted from mycelia grown on MEA plates with CTAB plant genome DNA fast extraction kit (Aidlab Biotechnologies Co., Ltd, Beijing, China) and stored at -20 °C until further use. Five loci, including the 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers (ITS), a 200-bp intron of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), partial actin (*ACT*), beta-tubulin (*TUB2*) and chitin synthase 1 (*CHS-1*), were amplified using the primer pairs ITS1/ITS4 (White et al. 1990; Gardes and Bruns 1993), GDF1/GDR1 (Guerber et al. 2003), ACT-512F/ACT-783R (Carbone and Kohn 1999), T1/Bt2b (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997) and CHS-79F/CHS-345R (Carbone and Kohn 1999), respectively.

PCR amplification and sequencing followed the protocols of Liu et al. (2017a). PCR amplicons were purified and sequenced at BGI Tech Solutions (Beijing Liuhe) Co., Limited (Beijing, China). Forward and reverse were assembled to obtain a consensus sequence with DNAMAN (v. 6.0.3.99; Lynnon Biosoft). Sequences generated in this study were deposited in GenBank (Table 1).

## Phylogenetic analysis

DNA sequences of concatenated *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2* loci were analysed to investigate the phylogenetic relationships amongst *Colletotrichum* species with DNA sequences available from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), as well as the sequences generated herein (Table 1). Multiple sequences were aligned using the MAFFT v.7.110 (http://mafft.cbrc.jp/alignment/server/) and adjusted manually in MEGA v.7.0 (Kumar et al. 2016). Gaps were manually adjusted to optimise the alignment (Tamura et al. 2013).

Phylogenetic analyses of Maximum Likelihood (ML), Bayesian Inference (BI) and Maximum Parsimony (MP) were performed. ML analyses were constructed on the RAxML-HPC BlackBox 8.2.10 (Stamatakis 2014) using the GTR+GAMMA model with 1000 bootstrap replicates. The Bayesian phylogenetic analysis was performed using a Markov Chain Monte Carlo (MCMC) algorithm in MrBayes v. 3.2.6 (Ronquist et al. 2012). Four MCMC chains were run from random trees for 2,000,000 generations and trees were sampled by each 1,000$^{th}$ generation. The first 25% of the trees of MCMC sampling were discarded as burn-in and posterior probabilities (PP) were determined from the remaining trees. Maximum Parsimony (MP) analysis, based on the concatenated dataset, was conducted in PAUP* v. 4.0b10 with the default options (Swofford 2002). Ambiguous regions in the alignment were excluded and gaps were treated as missing data. Clade stability was evaluated in a bootstrap analysis with 1,000 replicates with Maxtrees set to 1,000 and other default parameters implemented in PAUP* (Hillis and Bull 1993). Other measurements calculated parsimony scores including consistency index (CI), rescaled consistency (RC), homoplasy index (HI) and retention index (RI). The phylogenetic trees were configured in FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and edited using Adobe Illustrator CC2020 (Adobe Systems Inc., USA).

Sequences were analysed using the GCPSR model by performing a pairwise homoplasy index (PHI) test as described by Quaedvlieg et al. (2014) for the phylogenetically close, but not clearly delimited species. The PHI test was performed in SplitsTree v. 4.19.1 (Huson and Bryant 2006) to determine the recombination level within phylogenetically closely-related species using a five-locus concatenated dataset (*ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2*). If the resulting pairwise homoplasy index was below a 0.05 threshold ($\Phi w < 0.05$), it was indicative of significant recombination in the dataset. The relationship between closely-related species was visualised by constructing a split graph (Chethana et al. 2021; Jayawardena et al. 2021; Peng et al. 2023).

## Pathogenicity tests and virulence on walnut tissues

All isolated species were tested for their pathogenicity on walnut fruits and leaves. Isolates of all species were incubated on MEA plates for 7 days prior to inoculation. Spore suspension of isolates of *Colletotrichum juglandicola*

**Table 1.** GenBank accession numbers of isolates included in this study (newly-generated sequences are in bold). * = ex-type or authentic culture, (*) = ex-type or authentic culture of synonymised taxon and N/A = not available. Newly-generated sequences are indicated in bold.

| Taxon | Isolate designation | Host | Location | GenBank accession number(s) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | ITS | GAPDH | CHS-1 | ACT | TUB2 |
| *Colletotrichum aenigma* | ICMP 18608* | *Persea americana* | Israel | JX010244 | JX010044 | JX009774 | JX009443 | JX010389 |
| *C. aeschynomenes* | ICMP 17673* | *Aeschynomene virginica* | USA | JX010176 | JX009930 | JX009799 | JX009483 | JX010392 |
| *C. alatae* | CBS 304.67* | *Dioscorea alata* | India | JX010190 | JX010190 | JX009837 | JX009471 | JX010383 |
| *C. alienum* | ICMP 12071* | *Malus domestica* | New Zealand | JX010251 | JX010028 | JX009882 | JX009572 | JX010411 |
| *C. aotearoa* | ICMP 18537* | *Coprosma* sp. | New Zealand | JX010205 | JX010005 | JX009853 | JX009564 | JX010420 |
| *C. arecicola* | CGMCC 3.19667* | *Areca catechu* | China | MK914635 | MK935455 | MK935541 | MK935374 | MK935498 |
| *C. artocarpicola* | MFLUCC 18-1167* | *Artocarpus heterophyllus* | Thailand | MN415991 | MN435568 | MN435569 | MN435570 | MN435567 |
| *C. asianum* | ICMP 18580* | *Coffea arabica* | Thailand | FJ972612 | JX010053 | JX009867 | JX009584 | JX010406 |
| *C. australianum* | VPRI 43075* | *Citrus sinensis* | Australia | MG572138 | MG572127 | MW091987 | MN442109 | MG572149 |
| *C. boninense* | MAFF 305972* = CBS 123755 | *Crinum asiaticum* var. *sinicum* | Japan | JQ005153 | JQ005240 | JQ005327 | JQ005501 | JQ005588 |
| *C. camelliae* | CGMCC 3.14925 | *Camellia sinensis* | China | KJ955081 | KJ954782 | MZ799255 | KJ954363 | KJ955230 |
| *C. changpingense* | MFLUCC 15-0022 = CGMCC 3.17582* | *Rhizome of Fragaria × ananass* | China | KP683152 | MZ664048 | KP852449 | KP683093 | MZ673952 |
| *C. chiangmaiense* | MFLUCC 18-0945* | *Magnolia garrettii* | Thailand | MW346499 | MW548592 | MW623653 | MW655578 | N/A |
| *C. chrysophilum* | CMM 4268* | *Musa* sp. | Brazil | KX094252 | KX094183 | KX094083 | KX093982 | KX094285 |
| *C. cigarro* | ICMP 18539* | *Olea europaea* | Australia | JX010230 | JX009966 | JX009800 | JX009523 | JX010434 |
| *C. citrulli* | CAASZT54 | *Citrullus lanatus* | China | MZ475134 | OL456686 | OL901154 | OL449284 | OL456645 |
| | CAASZT52 | *Citrullus lanatus* | China | MZ475133 | OL456685 | OL901153 | OL449283 | OL456644 |
| *C. clidemiae* | ICMP 18658* | *Clidemia hirta* | USA, Hawaii | JX010265 | JX009989 | JX009877 | JX009537 | JX010438 |
| *C. cobbittiense* | BRIP 66219* | *Cordyline stricta* × *C. australis* | Australia | MH087016 | MH094133 | MH094135 | MH094134 | MH094137 |
| *C. conoides* | CGMCC 3.17615 | *Chili pepper* | China | KP890168 | KP890162 | KP890156 | KP890144 | KP890174 |
| *C. cordylinicola* | ICMP 18579* | *Cordyline fruticosa* | Thailand | JX010226 | JX009975 | JX009864 | HM470235 | JX010440 |
| *C. dimorphum* | CGMCC 3.16083* | *Ageratina adenophora* | China | OK030867 | OK513670 | OK513566 | OK513606 | OK513636 |
| | YMF 1.07303 | *Ageratina adenophora* | China | OK030866 | OK513669 | OK513565 | OK513605 | OK513635 |
| *C. dracaenigenum* | MFLUCC 19-0430* | *Dracaena* sp. | Thailand | MN921250 | MT215577 | MT215575 | MT313686 | N/A |
| *C. endophyticum* | MFLUCC 13-0418* | *Pennisetum purpureum* | Thailand | KC633854 | KC832854 | MZ799261 | KF306258 | MZ673954 |
| *C. fici-septicae* | MFLU 19-27708* | *Capsicum annuum* | China | KP145441 | KP145413 | KP145385 | KP145329 | KP145469 |
| *C. fructicola* | ICMP 18581* | *Coffea arabica* | Thailand | JX010165 | JX010033 | JX009866 | FJ907426 | JX010405 |
| *C. fructivorum* | CBS 133125* | *Vaccinium macrocarpon* | Burlington | JX145145 | MZ664047 | MZ799259 | MZ664126 | JX145196 |
| *C. gloeosporioides* | IMI 356878* = ICMP 17821 | *Citrus sinensis* | Italy | JX010152 | JX010056 | JX009818 | JX009531 | JX010445 |
| | CBS 273.51(*) = ICMP 19121 | *Citrus limon* | Italy | JX010148 | JX010054 | JX009903 | JX009558 | N/A |
| | DAR 76936 = ICMP 18738 | *Carya illinoinensis* | Australia | JX010151 | JX009976 | JX009797 | JX009542 | N/A |
| | ICMP12939 | *Citrus* sp. | New Zealand | JX010149 | JX009931 | JX009747 | JX009462 | N/A |
| | CBS 119204 = ICMP 18678 | *Pueraria lobata* | USA | JX010150 | JX010013 | JX009790 | JX009502 | N/A |
| | ICMP 12066 | *Ficus* sp. | New Zealand | JX010158 | JX009955 | JX009888 | JX009550 | N/A |
| | ICMP 18730 | *Citrus* sp. | New Zealand | JX010157 | JX009981 | JX009861 | JX009548 | N/A |

| Taxon | Isolate designation | Host | Location | GenBank accession number(s) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | ITS | GAPDH | CHS-1 | ACT | TUB2 |
| *C. gloeosporioides* | ICMP 12938 | *Citrus sinensis* | New Zealand | JX010147 | JX009935 | JX009746 | JX009560 | N/A |
| | ICMP 18694 | *Mangifera indica* | South Africa | JX010155 | JX009980 | JX009796 | JX009481 | N/A |
| | ICMP 18695 | *Citrus* sp. | USA | JX010153 | JX009979 | JX009779 | JX009494 | N/A |
| | ICMP 18697 | *Vitis vinifera* | USA | JX010154 | JX009987 | JX009780 | JX009557 | N/A |
| *C. grevilleae* | CBS 132879* | *Grevillea* sp. | Italy | KC297078 | KC297010 | KC296987 | KC296941 | KC297102 |
| *C. grossum* | CGMCC 3.17614* | *Chili pepper* | China | KP890165 | KP890159 | KP890153 | KP890141 | KP890171 |
| *C. hebeiense* | MFLUCC 13-0726* | *Vitis vinifera* | China | KF156863 | KF377495 | KF289008 | KF377532 | KF288975 |
| *C. hederiicola* | MFLU 15-0689* | *Hedera helix* | Italy | MN631384 | N/A | MN635794 | MN635795 | N/A |
| *C. helleniense* | CBS 142418* | *Poncirus trifoliata* | Greece, Arta | KY856446 | KY856270 | KY856186 | KY856019 | KY856528 |
| *C. henanense* | CGMCC 3.17354* | *Camellia sinensis* | China | KJ955109 | KJ954810 | MZ799256 | KM023257 | KJ955257 |
| *C. horii* | NBRC 7478* | *Diospyros kaki* | Japan | GQ329690 | GQ329681 | JX009752 | JX009438 | JX010450 |
| *C. hystricis* | CBS 142411* | *Citrus hystrix* | Italy, Catania | KY856450 | KY856274 | KY856190 | KY856023 | KY856532 |
| *C. jiangxiense* | CGMCC 3.17361* | *Camellia sinensis* | China | KJ955149 | KJ954850 | MZ799257 | KJ954427 | OK236389 |
| *C. kahawae* | IMI 319418* | *Coffea arabica* | Kenya | JX010231 | JX010012 | JX009813 | JX009452 | JX010444 |
| *C. ledongense* | CGMCC3.18888* | *Quercus palustris* | China | MG242008 | MG242016 | MG242018 | MG242014 | MG242010 |
| *C. makassarense* | CBS 143664* | *Capsicum annuum* | Indonesia | MH728812 | MH728820 | MH805850 | MH781480 | MH846563 |
| *C. mengyinense* | SAUCC0702* | *Rosa chinensis* | China | MW786742 | MW846240 | MW883686 | MW883695 | MW888970 |
| *C. musae* | CBS 116870* | *Musa* sp. | USA | JX010146 | JX010050 | JX009896 | JX009433 | HQ596280 |
| *C. nanhuaensis* | CGMCC 3.18962* | *Ageratina adenophora* | China | OK030870 | OK513673 | OK513569 | OK513609 | OK513639 |
| | YMF 1.04990 | *Ageratina adenophora* | China | OK030871 | OK513674 | OK513570 | OK513610 | OK513640 |
| *C. nupharicola* | CBS 470.96* | *Nuphar lutea* subsp. *polysepala* | USA | JX010187 | JX009972 | JX009835 | JX009437 | JX010398 |
| *C. pandanicola* | MFLUCC 17-0571* | *Pandanaceae* | Thailand | MG646967 | MG646934 | MG646931 | MG646938 | MG646926 |
| *C. perseae* | CBS 141365* | *Avocado* | Israel | KX620308 | KX620242 | MZ799260 | KX620145 | KX620341 |
| *C. proteae* | CBS 132882* | *Protea* sp. | South Africa | KC297079 | KC297009 | KC296986 | KC296940 | KC297101 |
| *C. pseudotheobromicola* | MFLUCC 18-1602* | *Prunus avium* | China | MH817395 | MH853675 | MH853678 | MH853681 | MH853684 |
| *C. psidii* | CBS 145.29* | *Psidium* sp. | Italy | JX010219 | JX009967 | JX009901 | JX009515 | JX010443 |
| *C. queenslandicum* | ICMP 1778* | *Carica papaya* | Australia | JX010276 | JX009934 | JX009899 | JX009447 | JX010414 |
| *C. rhexiae* | CBS 133134* | *Rhexia virginica* | Sussex | JX145128 | MZ664046 | MZ799258 | MZ664127 | JX145179 |
| *C. salsolae* | ICMP 19051* | *Salsola tragus* | Hungary | JX010242 | JX009916 | JX009863 | JX009562 | JX010403 |
| *C. siamense* | ICMP 18578* | *Coffea arabica* | Thailand | JX010171 | JX009924 | JX009865 | FJ907423 | JX010404 |
| *C. syzygicola* | MFLUCC 10-0624* | *Syzygium samarangense* | Thailand | KF242094 | KF242156 | N/A | KF157801 | KF254880 |
| *C. tainanense* | CBS 143666* | *Capsicum annuum* | Taiwan | MH728818 | MH728823 | MH805845 | MH781475 | MH846558 |
| *C. temperatum* | CBS 133122* | *Vaccinium macrocarpon* | Bronx | JX145159 | MZ664045 | MZ799254 | MZ664125 | JX145211 |
| *C. tengchongense* | YMF 1.04950 | *Isoetes sinensis* | China | OL842169 | OL981264 | OL981290 | OL981238 | N/A |
| *C. theobromicola* | CBS 124945* | *Theobroma cacao* | Panama | JX010294 | JX010006 | JX009869 | JX009444 | JX010447 |
| *C. ti* | ICMP 4832* | *Cordyline* sp. | New Zealand | JX010269 | JX009952 | JX009898 | JX009520 | JX010442 |
| *C. tropicale* | CBS 124949* | *Theobroma cacao* | Panama | JX010264 | JX010007 | JX009870 | JX009489 | JX010407 |
| *C. viniferum* | GZAAS 5.08601* | *Vitis vinifera* cv. *Shuijing* | China | JN412804 | JN412798 | N/A | JN412795 | N/A |
| *C. vulgaris* | YMF 1.04940 | *Hippuris vulgaris* | China | OL842170 | OL981265 | OL981291 | OL981239 | N/A |
| *C. wuxiense* | CGMCC 3.17894* | *Camellia sinensis* | China | KU251591 | KU252045 | KU251939 | KU251672 | KU252200 |
| *C. xanthorrhoeae* | BRIP 45094* | *Xanthorrhoea preissii* | Australia | JX010261 | JX009927 | JX009823 | JX009478 | JX010448 |
| *C. xishuangbannaense* | MFLUCC 19-0107* | *Magnolia liliifera* | China | MW346469 | MW537586 | MW660832 | MW652294 | N/A |
| *C. yulongense* | CFCC 50818* | *Vaccinium dunalianum* var. *urophyllum* | China | MH751507 | MK108986 | MH793605 | MH777394 | MK108987 |

| Taxon | Isolate designation | Host | Location | GenBank accession number(s) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | ITS | GAPDH | CHS-1 | ACT | TUB2 |
| *C. yunanjiangensis* | CGMCC 3.18964* | *Ageratina adenophora* | China | OK030885 | OK513686 | OK513583 | OK513620 | OK513649 |
| ***C. peakense*** | **CGMCC3.24308*** | ***Juglans regia*** | **China** | **OQ263017** | **OQ282975** | **OR004795** | **OQ282968** | **OQ282982** |
| | **CGMCC3.24307** | ***Juglans regia*** | **China** | **OQ263016** | **OQ282974** | **OR004794** | **OQ282967** | **OQ282981** |
| ***C. juglandicola*** | **CGMCC3.24312*** | ***Juglans regia*** | **China** | **OQ263015** | **OQ282973** | **OR004793** | **OQ282966** | **OQ282980** |
| | **CGMCC3.24313** | ***Juglans regia*** | **China** | **OQ263018** | **OQ282977** | **OR004797** | **OQ282970** | **OQ282984** |
| | **CGMCC3.24310** | ***Juglans regia*** | **China** | **OQ263020** | **OQ282979** | **OR004799** | **OQ282972** | **OQ282986** |
| | **CGMCC3.24309** | ***Juglans regia*** | **China** | **OQ263021** | **OQ282978** | **OR004798** | **OQ282971** | **OQ282985** |
| | **CGMCC3.24311** | ***Juglans regia*** | **China** | **OQ263019** | **OQ282976** | **OR004796** | **OQ282969** | **OQ282983** |

(CGMCC3.24312) and *Colletotrichum peakense* (CGMCC3.24308) obtained in this study were used for pathogenicity testing.

The pathogenicity test was performed on detached living walnut fruits and leaves. Briefly, fruits and leaves were washed with sterilised water and surface sterilised with 75% ethanol for 1 min. The fruits and leaves were inoculated using the spore suspension and non-wound inoculation methods (Fu et al. 2019; Zhao et al. 2019b). For the spore suspension and non-wound method, an aliquot of 20 µl of spore suspension ($1.0 \times 10^6$ conidia per ml) was inoculated on to fruits and leaves without wounding them. Eight replicates were used for each isolate. Sterilised water was used as the negative control. The inoculated detached fruits and leaves were incubated under 25 °C with 12/12 h light/dark photoperiod. Pathogenicity was determined by measuring the lesion length of fruits and leaves after 10 days' incubation. Mean comparisons were conducted using Tukey's honest significant difference (HSD) test ($\alpha = 0.05$) in R (Version 3.2.2, R Inc. Auckland, NZL).To fulfil Koch's postulates, small pieces of infected tissue were plated on to MEA to re-isolate the fungal isolates, which were identified, based on morphology and DNA sequences.

## Results

### Phylogenetic analyses

The concatenated *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2* dataset (1,948 characters with 369 parsimony-informative characters) from 79 in-group isolates of *Colletotrichum gloeosporioides* species complex was used for phylogenetic analysis. The outgroup taxon was *C. boninense* CBS 123755. The heuristic search with random addition of taxa (1,000 replicates) generated 5,000 most parsimonious trees (Length = 1,313, CI = 0.673, HI = 0.327, RI = 0.854, RC = 0.575). The topologies obtained from the Maximum Parsimony, Maximum Likelihood and Bayesian analysis were comparable. In three analyses (ML, BI and MP), *Colletotrichum juglandicola* and *Colletotrichum peakense* are consistently sibling to all other species of *C. gloeosporioides* species complex (95/1/89 and 100/0.98/58) (Fig. 1). Additionally, only the ML tree is presented here, with ML, BI and MP values plotted on the branches (Fig. 1).

To exclude the possibility that species delimitation might be interfered by recombination amongst the genes used for phylogenetic analyses, the multi-locus (*ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2*) concatenated datasets were

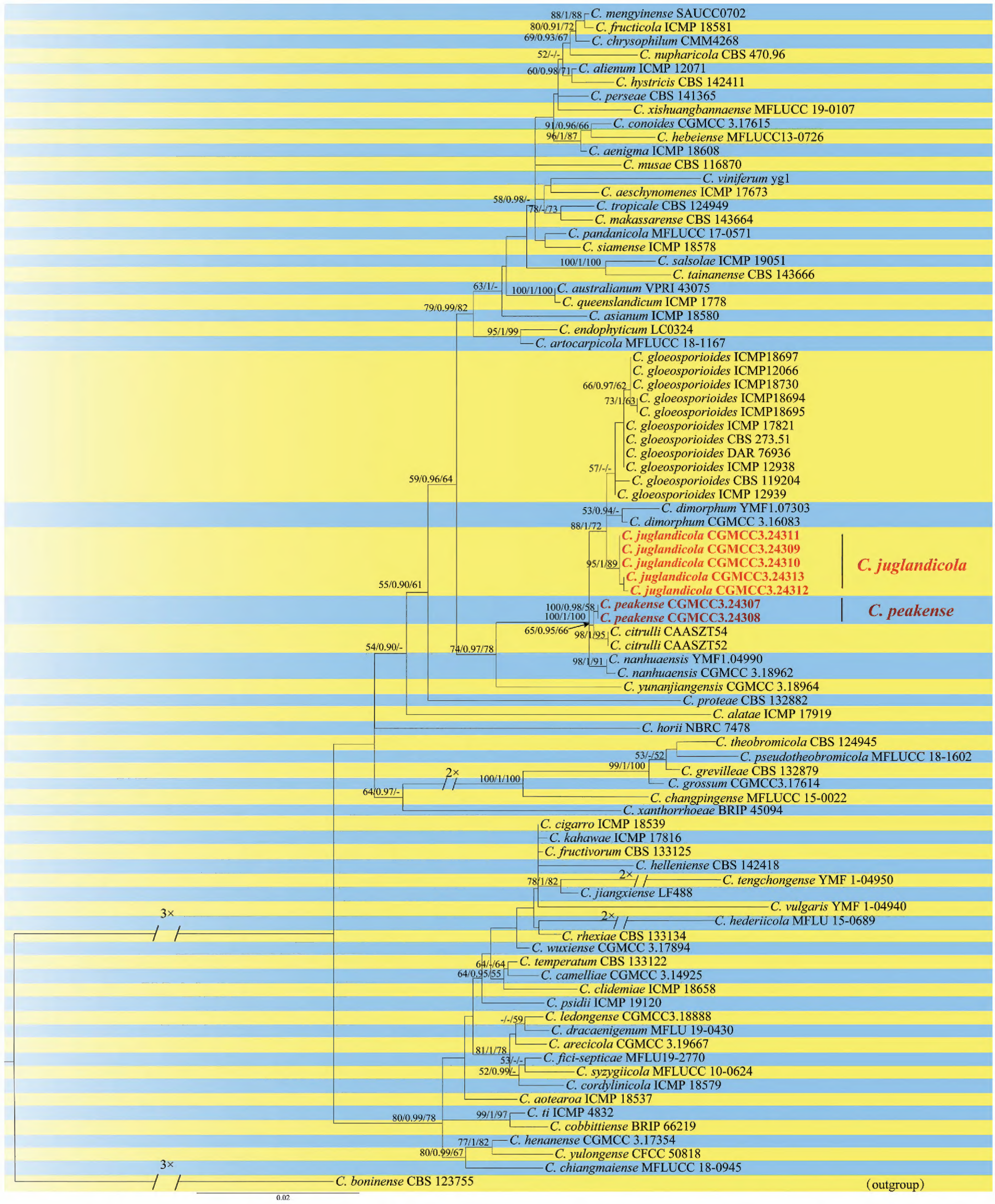


**Figure 1.** Phylogenetic tree of Maximum Likelihood analyses of 86 isolates in the *C. gloeosporioides* species complex. The species *C. boninense* (CBS 123755) was selected as an outgroup. The tree was built using concatenated sequences of *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2* genes. RAxML bootstrap support values (ML ≥ 50%), Bayesian posterior probability (PP ≥ 0.90) and MP bootstrap support values (ML ≥ 50%) are shown at the nodes (ML/PP/MP).

subjected to two PHI tests (Fig. 2) to determine the recombination level within phylogenetically closely-related species. The results showed that no significant recombination events were observed between *Colletotrichum juglandicola* and

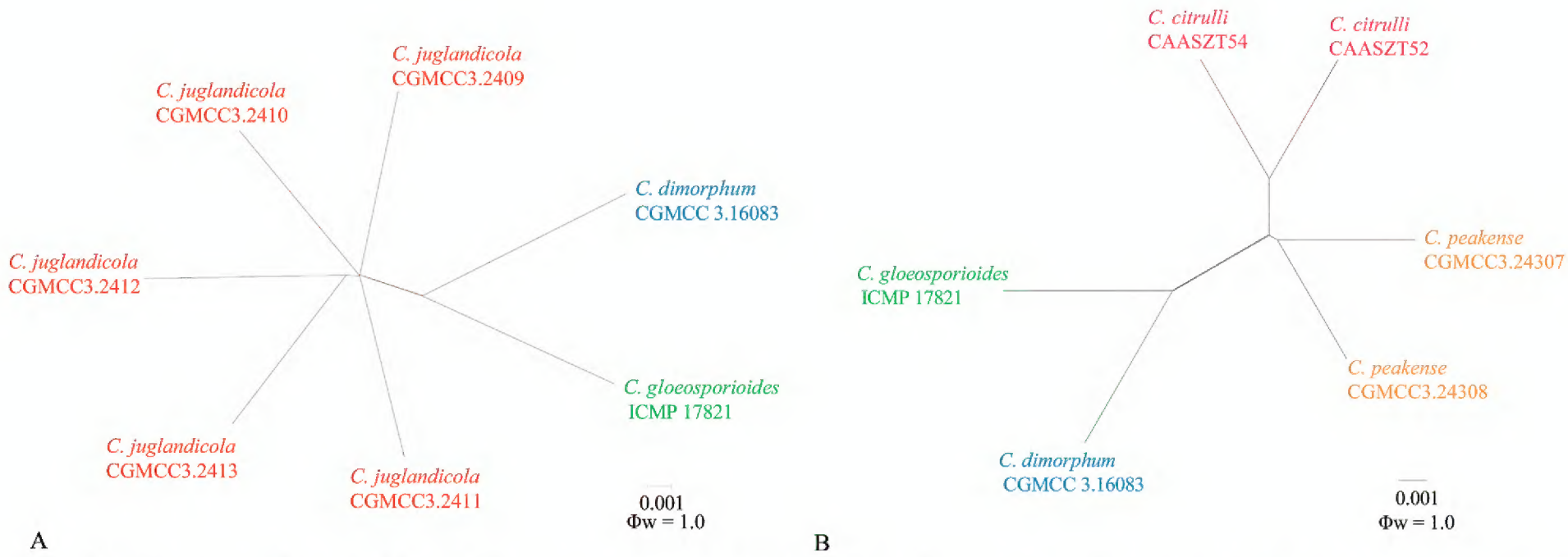


**Figure 2.** The result of the pairwise homoplasy index (PHI) tests of closely-related species using both LogDet transformation and splits decomposition **A, B** the PHI of *C. juglandicola* (**A**) or *C. peakense* (**B**) and their phylogenetically related isolates or species, respectively. PHI test value (Φw) < 0.05 indicate significant recombination within the dataset.

phylogenetically related isolates or species (*C. gloeosporioides* and *C. dimorphum*) (Fig. 2A) and between *C. peakense* and phylogenetically related species (*C. citrulli*, *C. gloeosporioides* and *C. dimorphum*) (Fig. 2B).

## Taxonomy

***Colletotrichum juglandicola*** **Y. Zhang ter. & L. Zhang, sp. nov.**
MycoBank No: 848731
Fig. 3

**Etymology.** Named from "*Juglans*", in reference to the host genus.

**Description.** ***Sexual morph*** not observed. ***Asexual morph*** developed on MEA. ***Conidiomata*** acervular, yellow to light brown, bearing conidial masses. ***Conidiophores*** hyaline, smooth-walled, septate, branched. ***Setae*** medium to dark brown, smooth to finely verruculose close to the tip, the tip rounded, 1–3 aseptate, 60–107.2 µm long. ***Conidiogenous cells*** 19.5–38.9 × 2.8–3.9 µm (mean SD = 28.6 ± 1.2 × 3.3 ± 0.1 µm, n = 20), subcylindrical, straight to curved. ***Conidia*** 14.6–20.0 × 4.2–6.6 µm (mean SD = 17.1 ± 1.0 × 5.2 ± 0.4 µm, L/W radio = 3.3, n = 100), hyaline, smooth-walled, subcylindrical, both ends round, 1–3-guttulate, contents granular. ***Appressoria*** 5–8.3 × 3.3–6.7 µm (mean SD = 6.3 ± 0.2 × 5.2 ± 0.2 µm, L/W radio = 1.2, n = 20), medium to dark brown, variable in shape, often smooth-walled, subglobose, ovate to broadly elliptical in outline.

**Culture characteristics.** Colonies on MEA, flat, with entire margin, hyaline, 65–72 mm diam. in 7 d. The colonies are round, white, the edges are flat and the aerial hyphae are lush. Myxospores are orange. The colony diameter reached 63–65 mm on PDA. The colonies are round, green-grey, the edges are flat and the aerial hyphae are lush.

**Additional specimens examined.** CHINA, Beijing, Changping District, Heishanzhai Village, from leaf of *Juglans regia* L., Y. Zhang and L. Zhang, 26 August 2021 (holotype HSG826-P5; ex-type living culture: CGMCC3.24312). CHINA, Beijing, Huairou District, Shuichangcheng Village, from leaf of *Juglans regia* L., Aug 2021, Y. Zhang and L. Zhang (Paratype SCCY826-22; living culture:

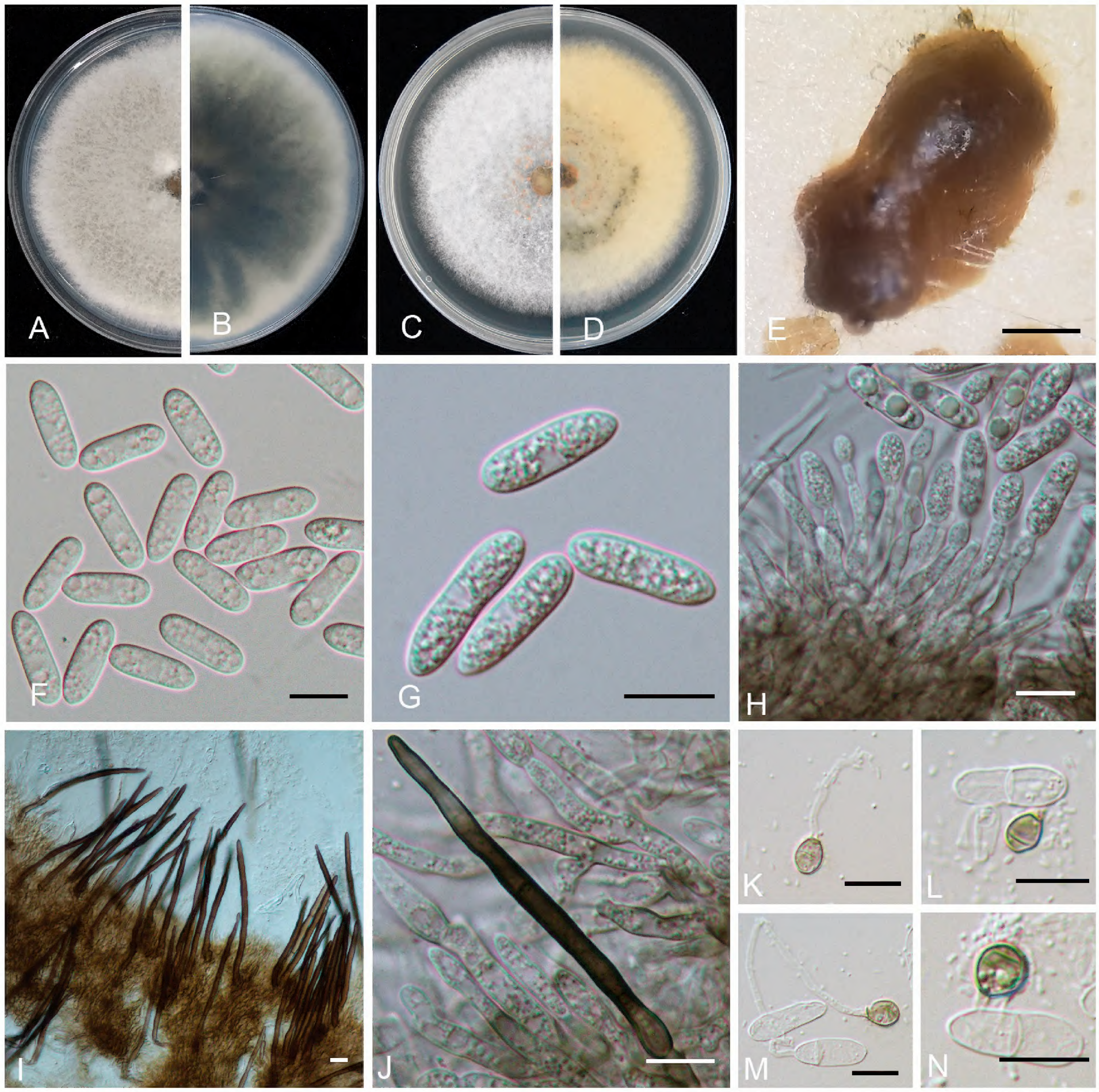


**Figure 3.** *Colletotrichum juglandicola* (from ex-type CGMCC3.24312) **A, B** colonies and reverse after 7 days on PDA medium **C, D** colonies and reverse after 7 days on MEA medium **E** conidiomata **F, G** conidia **H** conidiophores **I, J** setae **K–N** appressoria. Scale bars: 500 μm (**E**); 10 μm (**F–N**).

CGMCC3.24313). CHINA, Beijing, Haidian District, Jiufeng Village, from fruit of *Juglans regia* L., Aug 2021, Y. Zhang and L. Zhang (Paratype JFG826-P4; living culture CGMCC3.24311). CHINA, Beijing, Changping District, Yanshou Village, from fruit of *Juglans regia* L., Aug 2021, Y. Zhang and L. Zhang (Paratype YSG826-R1; living culture CGMCC3.24309). CHINA, Beijing, Changping District, Yanshou Village, from leaf of *Juglans regia* L., Aug 2021, Y. Zhang and L. Zhang (Paratype YSY826-2: living culture CGMCC3.24310).

**Notes.** Phylogenetic analysis of a concatenated five loci dataset indicated that the clade of *Colletotrichum juglandicola* nested in the clade of *C. gloeosporioides* species complex and was closely related, but indepen-

dent to *C. citrulli*, *C. dimorphum*, *C. gloeosporioides* and *C. nanhuaensis* (Cannon et al. 2008; Guo et al. 2022; Yu et al. 2022). *Colletotrichum citrulli* was reported from *Citrullus lanatus* (Cucurbitaceae) in China (Guo et al. 2022). Morphologically, *C. juglandicola* differed from *C. citrulli* by having longer conidia and setae and smaller appressoria (Table 2) (Guo et al. 2022). *Colletotrichum dimorphum* was reported from *Ageratina adenophora* (Asteraceae) in China (Guo et al. 2022). Morphologically, *C. juglandicola* differed from *C. dimorphum* by having setae, shorter appressoria and longer conidia (Yu et al. 2022) (Table 2). Morphologically, *C. juglandicola* differed from *C. gloeosporioides* or *C. nanhuaensis* by having longer conidia (Cannon et al. 2008; Guo et al. 2022) (Table 2). *Colletotrichum nanhuaensis* was reported from *Ageratina adenophora* (Asteraceae) in China (Guo et al. 2022) (Table 2). The PHI test ($\Phi w = 1.0$) detected no significant recombination between related isolates or species (Fig. 2A).

***Colletotrichum peakense*** **Y. Zhang ter. & L. Zhang, sp. nov.**

MycoBank No: 848730

Fig. 4

**Etymology.** Named after Beijing where the fungus was collected.

**Description.** ***Sexual morph*** not observed. ***Asexual morph*** developed on MEA. ***Conidiomata*** acervular, yellow, bearing conidial masses. ***Conidiophores*** hyaline, smooth-walled, septate and branched. ***Setae*** medium to dark brown, smooth to finely verruculose close to the tip, the tip rounded, 1–3 aseptate, 57.2–152.9 µm long. ***Conidiogenous cells*** 20–35.6 × 2.8–3.9 µm (mean SD = 26.1 ± 0.9 × 3.0 ± 0.1 µm, n = 20), subcylindrical, straight to curved. ***Conidia*** 13.5–20.5 × 3.1–5.9 µm (mean SD = 16.4 ± 1.4 × 4.9 ± 0.5 µm, L/W radio = 3.3, n = 100), hyaline, smooth-walled, subcylindrical, both ends round, 1–3-guttulate, contents granular. ***Appressoria*** 5.6–8.4 × 3.9–6.1 µm (mean

**Table 2.** Morphological comparison of species in the gloeosporioides species complex.

| Species | Type | Hosts | Distribution | Conidia (Mean ± SD) (µm) | Appressoria (µm) | Setae (µm) | Reference |
|---|---|---|---|---|---|---|---|
| *Colletotrichum citrulli* | Holotype | *Citrullus lanatus* | China | 16.2 ± 0.9 × 5.6 ± 0.5 | 8.0–12.0 × 6.0–10.0 | 42.0–79.0 | Guo et al. (2022) |
| *C. aenigma* | Holotype | *Persea americana* | Israel | 14.5 × 6.1 | 6.0–10.0 | Not observed | Weir et al. (2012) |
| *C. dimorphum* | Holotype | *Ageratina adenophora* | China | 14.6 ± 2 × 4.8 ± 0.7 | 5.7–10.6 × 5.0–9.0 | Not observed | Yu et al. (2022) |
| *C. fructicola* | Holotype | *Coffea arabica* | Thailand | 11.5 ± 1.0 × 3.6 ± 0.3 | 4.3–9.7 × 3.7–7.3 | Not observed | Prihastuti et al. (2009) |
| *C. gloeosporioides* | epitype | *Citrus sinensis* | Italy | 14.4 × 5.6 | 7.2–8.6 × 4.7–6.0 | 40.0–120.0 | Cannon et al. (2008) |
| ***C. juglandicola*** | **Holotype** | ***Juglans regia* L.** | **China** | **17.1 ± 1.0 × 5.2 ± 0.4** | **5–8.3 × 3.3–6.7** | 60.0–107.2 | **This study** |
| *C. kahawae* | Holotype | *Coffeae arabicae* | Kenya | 12.5–19.0 × 4.0 | 8.0–9.5 × 5.5–6.5 | Not observed | Waller et al. (1993) |
| *C. mengyinense* | Holotype | *Rosa chinensis* | China | 14.3 ± 1.1 × 5.3 ± 0.4 | Not observed | Not observed | Mu et al. (2021) |
| *C. nanhuaensis* | Holotype | *Ageratina adenophora* | China | 14.0 ± 1.1 × 5.4 ± 0.4 | 8.0–14.0 × 5.0–8.0 | 25.0 | Yu et al. (2022) |
| ***C. peakense*** | **Holotype** | ***Juglans regia* L.** | **China** | **16.4 ± 1.4 × 4.9 ± 0.5** | **5.6–8.4 × 3.9–6.1** | 57.2–152.9 | **This study** |
| *C. siamense* | Holotype | *Coffea arabica* | Thailand | 10.2 ± 1.7 × 3.5 ± 0.4 | 4.7–8.3 × 3.5–5.0 | Not observed | Prihastuti et al. (2009) |
| *C. viniferum* | Holotype | *Vitis vinifera* | China | 13.8 ± 1.0 × 5.4 ± 0.4 | 6.5–10.5 × 4.8–6.3 | Not observed | Peng et al. (2013) |

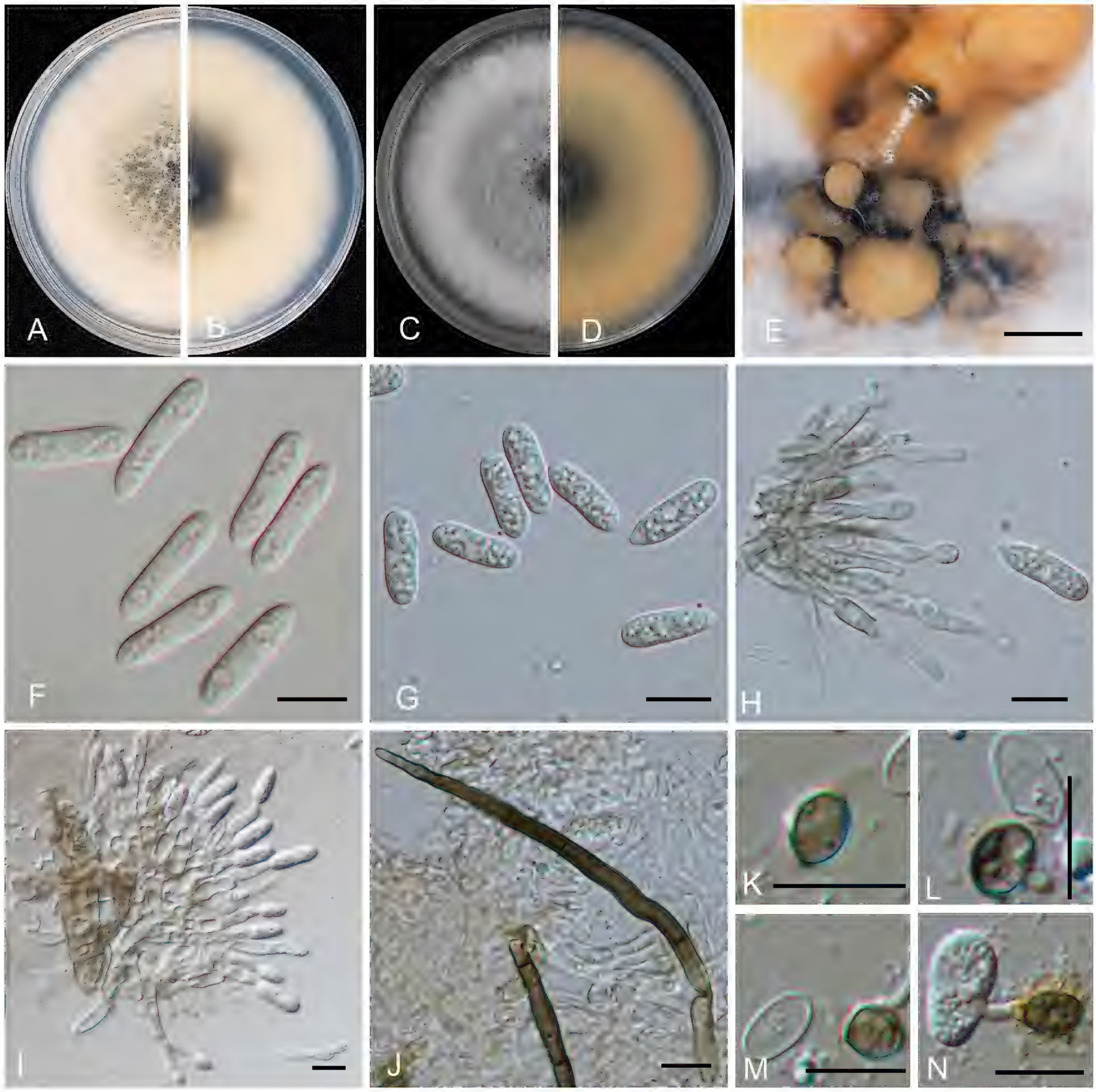


**Figure 4.** *Colletotrichum peakense* (from ex-type CGMCC3.24308) **A, B** colonies and reverse after 7 days on PDA medium **C, D** colonies and reverse after 7 days on MEA medium **E** conidiomata **F, G** conidia **H, I** conidiophores **J** setae **K–N** appressoria. Scale bars: 500 μm (**E**); 10 μm (**F–N**).

SD = 6.7 ± 0.2 × 5.1 ± 0.1 μm, L/W radio = 1.3, n = 20), medium to dark brown, variable in shape, often smooth-walled, subglobose, ovate to broadly elliptical in outline.

***Asexual morph*** developed on PDA. ***Conidia*** 14.7–22.2 × 4.1–6.3 μm (mean SD = 17.4 ± 1.6 × 5.2 ± 1.6 μm, L/W radio = 3.3, n = 50), hyaline, smooth-walled, subcylindrical, both ends round, 1–3-guttulate, contents granular.

**Culture characteristics.** Colonies on MEA, flat, with entire margin, hyaline, 68–78 mm diam. in 7 d. The colonies are round, aerial mycelium white or grey, floccose cottony; surface and reverse grey in the centre and white margin. Myxospores are orange. The colony diameter reached 76–80 mm on PDA. The

colonies are round, aerial mycelium white or grey, floccose cottony; surface and reverse grey in the centre and white margin.

**Additional specimens examined.** CHINA, Beijing, Changping District, Heishanzhai Village, from leaf of *Juglans regia* L., 26 Aug 2021, Y. Zhang and L. Zhang (holotype HSY826-18; ex-type living culture, CGMCC3.24308. CHINA, Beijing, Changping District, Heishanzhai Village, from leaf of *Juglans regia* L., 26 Aug 2021, Y. Zhang and L. Zhang (Paratype HSY826-18): living culture, CGMCC3.24307.

**Notes.** Phylogenetic analysis of a concatenated five loci dataset indicated that the clade of *Colletotrichum peakense* nested in the clade of *C. gloeosporioides* species complex and was closely related, but independent to *C. citrulli*, *C. dimorphum*, *C. gloeosporioides* and *C. nanhuaensis* (Cannon et al. 2008; Guo et al. 2022; Yu et al. 2022). Morphologically, *Colletotrichum peakense* was distinguishable from *C. citrulli* by having longer setae and smaller appressoria (Guo et al. 2022) (Table 2), while from *C. dimorphum* by having longer conidia and longer setae (Yu et al. 2022) (Table 2), from *C. gloeosporioides* by having longer conidia (Cannon et al. 2008) (Table 2) and from *C. nanhuaensis* by having longer conidia and shorter appressoria (Guo et al. 2022) (Table 2). The PHI test ($\Phi w = 1.0$) detected no significant recombination between related isolates or species-related species (Fig. 2B).

## Pathogenicity tests on walnut tissues

Pathogenicity tests were conducted to confirm Koch's postulates on fruits and leaves of walnut for *C. juglandicola* and *C. peakense*. The symptom of circular, necrotic, sunken lesions on fruits and as circular, necrotic lesions on leaves after 10 days of inoculation with typical orange conidial masses were observed from the inoculated site, whereas all control fruits and leaves remained healthy (Fig. 5). For spore suspension and non-wound methods, both on fruits and leaves, the lesion diameter of *C. peakense* was significantly higher than *C. juglandicola* ($P < 0.05$) (Table 3). Furthermore, *Colletotrichum* isolates could consistently be re-isolated from symptomatic lesions, but never from control. Koch's postulates were performed by successful pathogen re-isolation from all the necrotic fruits and leaves. The morphology and DNA sequences of these new isolates were consistent with the initial inoculation.

**Table 3.** Pathogenicity of *Colletotrichum juglandicola* (CGMCC3.24312) and *Colletotrichum peakense* (CGMCC3.24308) on walnut fruits and leaves using spore suspension as inoculum 10 days after inoculation.

| Species | Walnut fruits inoculated with Spore suspension and non-wound ± SD (mm) | Walnut leaves inoculated with Spore suspension and non-wound ± SD (mm) |
|---|---|---|
| *Colletotrichum juglandicola* | 5.80 ± 1.27 b | 8.90 ± 2.28 b |
| *C. peakense* | 9.50 ± 1.0 a | 16.79 ± 2.58 a |
| Non-inoculated control | 0 ± 0 c | 0 ± 0 c |

Note: Data followed by different letters in each column are significantly different, based on HSD tests at the $P < 0.05$ level.

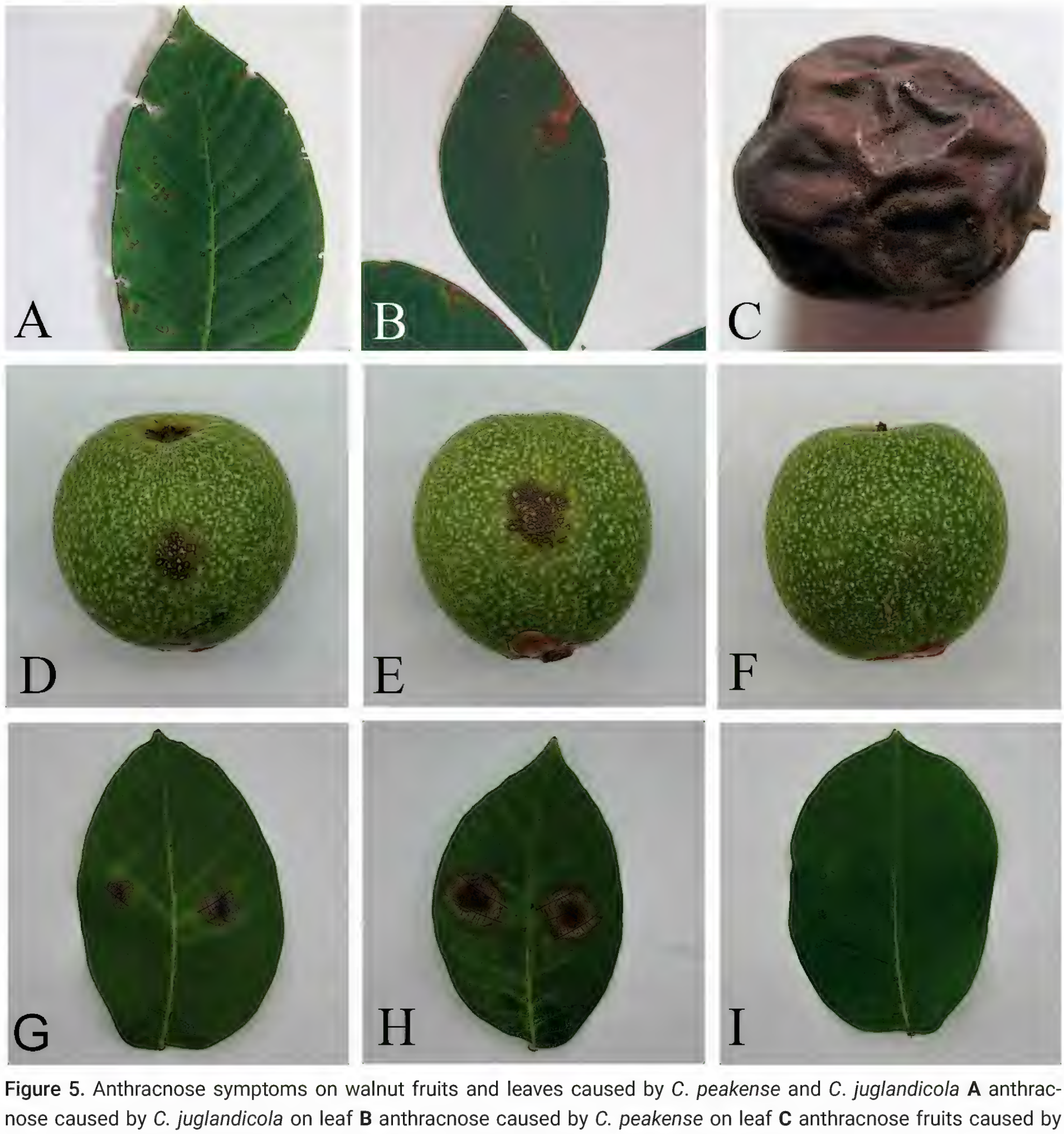


**Figure 5.** Anthracnose symptoms on walnut fruits and leaves caused by *C. peakense* and *C. juglandicola* **A** anthracnose caused by *C. juglandicola* on leaf **B** anthracnose caused by *C. peakense* on leaf **C** anthracnose fruits caused by *C. juglandicola* **D, G** symptoms of *C. juglandicola* (CGMCC3.24312) using spore suspension and non-wound inoculation methods after 10 days inoculation on walnut fruit (**D**) and leaf (**G**) **E, H** symptoms of *C. peakense* (CGMCC3.24308) using spore suspension and non-wound inoculation methods after 10 days inoculation on walnut fruit (**E**) and leaf (**H**) **F, I** symptoms resulting from sterilised water and non-wound inoculation methods after 10 days inoculation on walnut fruit (**F**) and leaf (**I**).

## Discussion

Phylogenetic analyses, based on five concatenated loci (*ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2*), indicated that either *Colletotrichum juglandicola* or *C. peakense* formed a distinct clade within the *C. gloeosporioides* complex, while sibling to other species (Fig. 1). On the phylogenetic tree, *C. juglandicola* is closely related to *C. citrulli*, *C. dimorphum*, *C. gloeosporioides* and *C. nanhuaensis* (Fig. 3).

Morphologically, *C. juglandicola* can be readily distinguished from *C. citrulli*, *C. dimorphum*, *C. gloeosporioides* and *C. nanhuaensis*, based on its longer conidial size, presence or absence of setae and appressoria size (Table 2). Phylogenetically, *C. peakense* is closely related to *C. citrulli*, *C. dimorphum*, *C. gloeosporioides* and *C. nanhuaensis* (Fig. 4). *Colletotrichum peakense* can be distinguishable from *C. citrulli*, *C. dimorphum*, *C. gloeosporioides* and *C. nanhuaensis* by its longer setae and smaller appressorial size (Table 2).

Thus far, 14 species of *Colletotrichum* have been reported from *Juglans regia* L., namely *C. acutatum*, *C. fioriniae*, *C. godetiae*, *C. juglandis* and *C. nymphaeae* of the Acutatum species complex, *C. aenigma*, *C. fructicola*, *C. gloeosporioides*, *C. kahawae*, *C. mengyinense*, *C. siamense* and *C. viniferum* of Gloeosporioides species complex, *C. liaoningense* of Magnum species complex and *C. sojae* of Orchidearum species complex (Simmonds 1966; Alvarez 1976; Gorter 1977; Pennycook 1989; Liu et al. 1995; Crous et al. 2000; Chen 2003; Cho and Shin 2004; Gadgil et al. 2005; Juhásová et al. 2005; Sreenivasaprasad and Talhinhas 2005; Kobayashi 2007; Qu et al. 2011; Damm et al. 2012; Zhu et al. 2014; Zhu et al. 2015; Wang et al. 2017, 2018; Da Lio et al. 2018; He et al. 2019; Savian et al. 2019; Wang et al. 2020; Wang et al. 2021, 2023; Luongo et al. 2022; Ma et al. 2022; Wei et al. 2022; Li et al. 2023), of which, *Colletotrichum acutatum*, *C. fioriniae*, *C. godetiae*, *C. juglandis* and *C. nymphaeae* differed from *C. juglandicola* and *C. peakense* by their acute-ended conidia (Damm et al. 2012). *Colletotrichum aenigma*, *C. fructicola*, *C. gloeosporioides*, *C. kahawae*, *C. mengyinense*, *C. siamense* and *C. viniferum* differed from *C. juglandicola* and *C. peakense*, by the size of conidia (Table 2). The conidia shape of *Colletotrichum juglandicola* and *C. peakense* was comparable to *C. liaoningense*, while the longer conidia and longer appressoria size were distinguishable from the latter (Diao et al. 2017) (Table 2). *Colletotrichum juglandicola* and *C. peakense* were distinguishable from *C. sojae* by their shorter appressoria (Damm et al. 2019) (Table 2).

Pathogenicity tests indicated that both *Colletotrichum juglandicola* and *C. peakense* cause anthracnose disease in walnut fruits and leaves. Both on fruits and leaves, the virulence of *C. peakense* was more severe than *C. juglandicola* ($P < 0.05$). *Colletotrichum gloeosporioides* had been reported more severe than most other species in Beijing, which was supported by the current study in that *C. gloeosporioides* was more severe than *C. juglandicola* (12.33 ± 0.29 mm in 4 days vs. 8.90 ± 2.28 mm in 10 days) (Li et al. 2023).

Both *Colletotrichum juglandicola* and *C. peakense* belong to the *C. gloeosporioides* species complex, which has been reported as one of the most important pathogens worldwide and has infected at least 1,000 plant species (Phoulivong et al. 2010). *Colletotrichum gloeosporioides* species complexes could be either broad or narrow host ranges (Crouch et al. 2014; Liu et al. 2022). It appears that some species of *Colletotrichum*, such as *C. horii* (on persimmon) and *C. kahawae* (on coffee) may be restricted to certain hosts genera or families, while some others may have a wide range of hosts. For instance, *C. fructicola* has been reported from walnut, coffee, chilli, longan, shine muscat, papaya and tea (Prihastuti et al. 2009; Yang et al. 2009; Sharma and Shenoy 2014; Wang et al. 2018; Lim et al. 2020; Lin et al. 2021). To summarise, in Beijing, *C. juglandicola* and *C. peakense*, two species new to science, were the causal agents of walnut anthracnose.

## Additional information

### Conflict of interest

The authors have declared that no competing interests exist.

### Ethical statement

No ethical statement was reported.

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### Author contributions

YZ designed the experiments. YZ, LZ and LLZ prepared the samples, conducted the molecular experiments, and analyzed the data. LZ drafted the manuscript. YZ, LZ, YQY, LLZ, YQX and JH revised the manuscript. All authors contributed to the article and approved the submitted version.

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### Data availability

All of the data that support the findings of this study are available in the main text.

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